Smart delivery of antitumoral platinum complexes from biomimetic hydroxyapatite nanocrystals

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This study widens the role of biomimetic hydroxyapatite (HA) nanocrystals as bone substitutes and describes how they can be used as bone-specific drug delivery devices for in situ treatment of bone tumors upon local implantation. The adsorption and release kinetics of bis-{ethylenediamineplatinum(II)}-2-amino-1-hydroxyethane-1,1-diyly-bisphosphonate and bis-{ethylenediamineplatinum(II)}medronate on two kinds of HA nanocrystals having different morphologies, crystallinity degrees and surface areas have been investigated. The different chemical structures of the two Pt complexes appreciably affect not only the affinity towards the two kinds of HA, but also their release. The Pt complex loading is slightly greater for the HA characterized by lower crystallinity and higher surface area, with respect to the more crystalline one. The cytotoxicity of Pt complexes released from the HA were tested against human cervix carcinoma cells and, interestingly, were found to be more cytotoxic than the unmodified complexes. The released Pt species are therefore the active dichloroethyleneplatinum(II) or related solvato species formed by Pt-bisphosphonate bond breaking.

Introduction

Calcium phosphate is the inorganic component of many biological hard tissues, the most prominent ones being bones and teeth. In bone and dentin, the mineral phase consists of platelet-like hydroxyapatite (HA) crystals of a few nanometers in thickness and some tens of nanometers in length. The synthesis and chemico-physical characterization of such in vitro prepared nanocrystals not only allow us to learn more about the chemical process of formation of this mineral in vivo, but also to prepare new biomaterials for bone graft purposes that closely resemble the natural ones. Numerous synthetic strategies exist for producing HA and ionic substituted HA including wet precipitation, hydrothermal and ultrasonic nebulization methods, electrodeposition, sol-gel and solid state synthesis. Dimensions, morphology, crystallinity degree and surface properties represent the physico-chemical features which should be tailored in synthetic HA crystals for optimising their specific biomedical applications. Hydroxyapatite is also known for its capability to bind a wide variety of molecules and most therapeutic agents for bone diseases under physiological conditions. A main challenge for innovative bone substitute biomaterials is their functionalization with bioactive molecules which can transfer information and act specifically on the biological environment. In other words, the implanted biomaterial can act as a local scaffold for cell invasion and formation of functional tissue and, in the meantime, deliver previously loaded biomolecules. In this way HA nanocrystals can enhance their osteointegration or osteoinduction properties and also stimulate specific cellular responses at a molecular level. The adsorption and release properties of bioactive molecules are strongly influenced not only by the chemical properties of the drug molecule but also by the chemical and structural characteristics of the HA substrates.

Nowadays cisplatin is one of the five most used drugs in the treatment of solid tumors such as testicular, ovarian, and bladder carcinomas. However, the use of cisplatin is limited by some serious drawbacks such as nausea, vomiting, ototoxicity, myelotoxicity and concentration dependent nephrotoxicity. The systemic side effects of cisplatin and related Pt drugs can be lowered by several methods. A promising strategy makes use of carrier ligands able to promote a specific accumulation of the drug in the target tissue. The local treatment of the tumor by the implantation of a matrix in which the drug has been embedded could represent another appealing perspective.

Bisphosphonates (BPs) appear to be promising carrier ligands for targeting a drug to calcium-containing tissues, such as bone. They are characterized by two phosphate groups attached to a single carbon atom and have strong affinity for the Ca$^{2+}$ ions of bone apatite. Bisphosphonates are already used clinically to inhibit demineralization by osteoclasts and to shift the balance between mineralization and demineralization towards bone formation. They not only bind to hydroxyapatite, which is deposited by osteoclasts during bone formation, but are also able to inhibit some cellular mechanisms responsible for bone resorption, their anti-osteoclastic activity being due to inhibition of the...
enzyme farnesyl diphosphate synthase. On the basis of the previous consideration we came to the conclusion that platinum-bisphosphonate complexes could be loaded onto calcium-containing matrices to be used as bone fillers. Such composites could be administered locally at the site of an osteosarcoma. In fact, in the case of a bone tumor, after surgical removal, concealing the bone tissue loss is necessary and to this aim materials designed for bone grafting and replacement are required. Moreover, a subsequent anti-tumor treatment needs to be carried out to avoid recurrence. In this sense, the use of such bioactive and drug releasing materials seems to be attractive in order to perform both these features. They would act both as bone substitutes and as platinum drug releasing agents with the final goal of locally inhibiting the tumor re-growth and reducing the systemic toxicity.

Therefore in a previous work the adsorption and release of cisplatin, alendronate, and a platinum-medronate complex towards two bio-mimetic synthetic hydroxyapatite nanocrystals having different crystal shapes and chemico-physical properties were investigated. The results showed a high loading capacity of the synthetic hydroxyapatite for the platinum-bisphosphonate complex and the ability of the composite material to release the Pt compound to the surrounding medium in a slow and controlled manner. In addition to hydroxyapatite, calcium-containing silica polymers were also shown to have release-modulating properties for Pt-bisphosphonate complexes due to their calcium components. In particular, as with HA, it was found that Ca\(^{2+}\) acts as an anchoring moiety for the bisphosphonate ligand and influences the nature of the platinum complex released in solution. These results showed that the combination of calcium-containing matrices with Pt-bisphosphonate complexes results in more powerful delivery systems than those obtained by simple physical embedment of cisplatin in the matrices.

In the present study we have investigated the adsorption and release of two platinum complexes containing geminal bisphosphonates, differing in their charges and physico-chemical properties, towards two kinds of hydroxyapatite. The two HAs differed in the degrees of crystallinity, crystal morphologies and dimensions. The results show that the different chemical structures of the two platinum complexes influence both the loading and the releasing processes towards HA nanocrystals. In addition, the Pt complexes released by the HA matrices were preliminarily tested for their cytotoxic activity in human cultured cells. Spectroscopic and cytotoxicity studies are consistent with the hypothesis that the matrices are able to anchor the BP carrier ligand and the released Pt species is thus the active [PtCl\(_2\)](en) (en = ethylenediamine) in the matrices.

This study describes how hydroxyapatite nanocrystals can perform as bone-specific drug delivery devices to be used for treatment of bone tumors upon local implantation.

**Experimental**

**Materials**

Common high-purity chemical reagents were supplied from Sigma. Ultra-pure water (0.22 µS, 25 °C) was used in all experiments. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), F12-HAM’s medium, and penicillin/streptomycin solution were obtained from Sigma Chemical Co, St. Louis, USA. Foetal calf serum was purchased from Euroclone, Berlin, Germany.

**Synthesis of the platinum complexes**

Bis-[ethylenediamineplatinum(II)]-2-amino-1-hydroxyethane-1,1-diy-bisphosphonate (A), bis-[ethylenediamineplatinum(II)] medronate (B), and dichloridoethylenediamineplatinum(II) ([PtCl\(_2\)](en)), en = ethylenediamine were prepared as already reported in the literature. The purity of Pt complexes was higher than 95% as established by combustion analysis carried out with a Hewlett Packard 185 C, H, and N analyzer. Anal. calculated for [A] + HSO\(_4\), 2H\(_2\)O (C\(_3\)H\(_7\)2N\(_2\)O\(_3\)P\(_2\)Pt\(_2\)), MW = 861.47): C, 8.37; H, 3.16; N, 8.13%. Found: C, 8.66; H, 3.44; N, 7.89%. Anal. calculated for [B]·2H\(_2\)O (C\(_3\)H\(_7\)2N\(_2\)O\(_3\)P\(_2\)Pt\(_2\)), MW = 718.35): C, 8.36; H, 3.09; N, 7.80%. Found: C, 3.83; H, 2.96; N, 7.45%. Anal. calculated for [PtCl\(_2\)](en) (C\(_3\)H\(_7\)2N\(_2\)Cl\(_2\)Pt, MW = 326.08): C, 7.37; H, 2.47; N, 8.59%. Found: C, 7.31; H, 2.56; N, 8.50%. The elemental analyses and the spectroscopic characterization of the platinum complexes were consistent with data reported in the literature.

The two dinuclear Pt-bisphosphonate complexes A and B were found to be very stable in aqueous solution even after several months after the preparation of the samples as evidenced by NMR spectroscopy (data not shown).

**Synthesis of HA nanocrystals**

HA nanocrystals were synthesized in order to produce bonelike apatite crystals with different chemico-physical characteristics. Two kinds of HA nanocrystals were prepared: acicular shaped poorly crystalline HA (HAp) and plate shaped highly crystalline HA (HAc).

HAp nanocrystals were synthesized as previously reported. HAc nanocrystals were prepared from an aqueous suspension of Ca(OH)\(_2\) (1.35 M, 1 L) by slow addition of aqueous H\(_3\)PO\(_4\) (1.26 M, 600 mL), at 95 °C, under nitrogen atmosphere. The reaction mixture was kept under stirring (always at 95 °C and under N\(_2\) atmosphere) for 24 hours, then stirring was suspended and the mixture was left standing for 2 hours to allow deposition of the inorganic phase. This latter was isolated by filtration of the mother liquor, repeatedly washed with water and freeze-dried at −60 °C under vacuum (3 mbar) for 24 h.

The HAp and HAc granular fractions having dimensions ranging from 100 to 150 µm were selected for the study.

**Morphological characterization of HA**

Transmission electron microscopy (TEM) investigations were carried out using a Philips CM 100 instrument (80 kV). The powdered samples were ultrasonically dispersed in ultrapure water and then a few droplets of the slurry deposited on holey-carbon foils supported on conventional copper microgrids.

**Structural characterization of HA**

X-Ray diffraction powder patterns were collected using an Analytical X’Pert Pro equipped with an X’Celerator detector powder diffractometer using Cu K\(_\alpha\) radiation generated at 40 kV and 40 mA. The instrument was configured with 1/2° divergence.
and receiving slits. A quartz sample holder was used. The 20 range was from 5° to 60° with a step size (°29) of 0.05 and a counting time (s) of 3.

The degree of HA crystallinity was calculated according to the formula:\(^{33}\)

\[
\text{Crystallinity [\%]} = 100 \cdot \frac{C}{A + C}
\]

where C is the area from the peaks in the diffraction pattern (“the crystalline area”) and A is the area between the peaks and the background (“the amorphous area”).

Crystal domain sizes along the HA axis directions were calculated applying the Scherrer equation: \(^{32}\)

\[
L_{(lai)} = \frac{0.94\lambda}{\cos\theta \sqrt{\Delta^2 - \Delta_0^2}}
\]

where \(\theta\) is the diffraction angle for plane (hkl), \(\Delta r\) and \(\Delta_0\) the widths in radians of reflection (hkl) at half height for the synthesized and the reference HA materials, respectively, and \(\lambda = 1.5405 \text{ Å}\).

**Surface characterization of HA**

Specific surface area measurements were undertaken using a Carlo Erba Sorpty 1750 instrument by measuring \(\text{N}_2\) adsorption at 77 K and adopting the well-known BET procedure. \(^{33}\)

X-ray photoemission spectroscopy (XPS) analyses were performed in an M-Probe Instrument (SSI) equipped with a monochromatic Al K\(\alpha\) source (1486.6 eV) with a spot size of 200 \(\times\) 750 µm and pass energy of 25 eV, providing a resolution for 0.74 eV. With a monochromatic source, an electron flood gun was used to compensate the build up of positive charge on the insulator samples during the analyses. 10 eV electrons were selected to perform measurements on these samples. The accuracy of the reported binding energies (BE) was estimated to be \(\pm 0.2\) eV. The quantitative data were also accurately checked and reproduced several times (at least ten times for each sample) and the percentage error was estimated to be \(\pm 1\%\).

**Spectroscopic and photometric analyses of HA**

Calcium, platinum and phosphorus contents were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES, Liberty 200, Varian, Clayton South, Australia). Samples were dissolved in 1% wt ultrapure nitric acid. The following analytical wavelengths were chosen: Ca 422 nm, P 213 nm and Pt 265 nm.

**Platinum complexes spectrophotometric determination**

The complexes A and B were measured at 225 nm (\(\varepsilon = 4.25 \times 10^4\) M \(-1\) cm \(-1\)) and 226 nm (\(\varepsilon = 2.38 \times 10^4\) M \(-1\) cm \(-1\)) respectively against the reagent blank, using a Cary 300 BIO UV-Vis spectrophotometer (Varian, Palo Alto CA).

**Platinum complexes adsorption onto HA nanocrystals**

An aliquot (1.5 mL) of platinum complex solution (1 mg/mL) was added to 10 mg HA in a 2 mL conical polyethylene Eppendorf tube. After 15 s of treatment in a vortex apparatus, the HA suspension was maintained in a bascule bath at 37 °C. The adsorption profile for the platinum complex was determined by measuring the concentration of Pt complex remaining in the supernatant solution as function of time. At scheduled times, aliquots (100 µL) of the supernatant (that was well separated from the solid phase by 3 min of centrifugation at 10000 rpm on a micro Centrifuge 4214) were removed for Pt complex quantification and replaced with fresh water. The complexes A and B were measured by UV-Vis as described above.

In order to evaluate the physiosorbed platinum complex with respect to the chemisorbed one, the HA-platinum complex conjugates were washed twice using 1 mL of ultrapure water. The platinum complex in the washing water was quantified by ICP-OES analysis, therefore the residual drug (calculated as the difference between the total adsorbed drug and the drug removed by washing) was considered to be effectively bound to the HA nanocrystals.

**Platinum complexes and Ca\(^{2+}\) release from HA nanocrystals**

The solid apatite/complex conjugates were washed twice with ultrapure water and freeze-dried for 48 hours at –60 °C under vacuum (3 mbar). An aliquot (about 5 mg) of the conjugate was mixed in a polyethylene tube with Hepes buffer saline solution (10 mM, 10 mL) (containing chloride ions in the range 0.18–0.20 M). After 15 s of treatment in a vortex apparatus, the HA suspension was maintained in a bascule bath at 37 °C. At scheduled times, aliquots (1 mL) of the supernatant (that was well separated from the solid phase by 6 min of centrifugation at 5000 rpm on a THERMO multi-speed PK 121) were removed for platinum and calcium ion quantification and replaced with fresh buffer. The supernatant was analyzed by UV-Vis spectroscopy and compared with the [PtCl\(_2\)(en)] UV-vis spectrum. The amounts of platinum and calcium ions released from the HA nanocrystals were determined by ICP measurements.

**Experiments with human cancer cells**

Platinum complexes were dissolved in F12-HAM’s medium just before the experiment. Aliquots (about 10 mg) of the solid apatite/complex conjugates were mixed in a polyethylene tube with F12-HAM’s medium. After 15 s of treatment in a vortex apparatus, the HA suspension was maintained in a bascule bath at 37 °C. After 24 h, the supernatant (separated as described above) was removed and utilized for cytotoxicity studies. Platinum released from the HA nanocrystals was determined by ICP measurements.

HeLa cervix squamous carcinoma cell line (ATCC, Rockville, MD) was maintained in the logarithmic phase at 37 °C in a 5% carbon dioxide atmosphere in F-12 HAM’s medium containing 10% foetal calf serum (Biochrom-Seromed GmbH&Co, Berlin, Germany), antibiotics (50 units mL \(^{-1}\) penicillin and 50 µg mL \(^{-1}\) streptomycin) and 2 mM L-glutamine.

**Cytotoxicity assay**

The growth inhibitory effect toward HeLa cells was evaluated by means of MTT (tetrazolium salt reduction) assay. \(^{34}\) Briefly, 5 \(\times\) 10\(^4\) cells were seeded in 96-well microplates in growth medium
(100 μL) and then incubated at 37 °C in a 5% carbon dioxide atmosphere. After 24 h, the medium was removed and replaced with a fresh one containing the compound to be studied. Triplicate cultures were established for each treatment. After 72 h in the dark, each well was treated with 10 μL of a 5 mg mL⁻¹ MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) saline solution, and after 5 h of incubation, 100 μL of a sodium dodecylsulfate (SDS) solution in HCl (0.01 M) was added. After overnight incubation, the inhibition of cell growth induced by the tested compounds was detected by measuring the absorbance of each well at 570 nm using a Bio-Rad 680 microplate reader (Milan, Italy). The mean absorbance for each drug dose was expressed as a percentage of the control untreated well absorbance and plotted vs. drug concentration.

Since platinum drugs can be light sensitive all incubations were carried out in the dark.

**Statistical analysis**

Determinations of HA crystallite domain size along the c direction, bulk and surface Ca/P ratio and specific surface area were carried out 5 times on the same synthesis product. Data are presented as mean value ± SD. Adsorption, release and cytotoxic experiments were performed in triplicate, and results are reported as mean values ± SD. Data obtained from experiments were compared by a two tailed t-test. Differences were considered statistically significant at a significance level of 90%.

**Results and discussion**

**Synthesis and chemico-physical characterization of HA nanocrystals**

Two different procedures were used in order to synthesize biomimetic HA nanocrystals with different chemico-physical properties. Powder X-ray diffraction patterns of HAp and HAhc (Fig. 1) show the characteristic diffraction maxima of hydroxyapatite single phase (JCPDS 9-432).

The diffraction pattern of HApc exhibits not well defined diffraction maxima indicating a relatively low degree of crystallinity. In contrast, HAhc exhibits a slightly higher degree of crystallinity resulting from the higher temperature of synthesis and aging (95 °C). The HAp and HAhc degrees of crystallinity were quantified according to a previously reported method¹⁴ (Table 1). The crystal domain sizes along the c-axis and along a perpendicular to it, were calculated using Scherrer’s formula (see experimental section) using the 2θ = 26° (002) and 2θ = 39° (310) diffraction peaks, respectively (Table 1). The crystallite domain sizes of the nanocrystals synthesised at mild temperature (HAp) appeared slightly shorter than the domain sizes of HAhc.

The surface Ca/P ratio determined by X-ray photoemission spectroscopy (XPS) analysis was significantly lower than the bulk Ca/P ratio as a result of nanocrystalline apatite surface disorder.²⁹ Due to an increasing crystal size, the specific surface area of the HAp nanocrystals was slightly higher than that determined for the HAhc particles (Table 1).

The transmission electron microscopy images of HAp and HAhc reveal that they have accicular and plate-like morphologies, respectively, while the length dimension of both HAs ranges between 80 and 120 nm (Fig. 2).

**Platinum complexes adsorption onto HA**

Bis-(ethylenediamineplatinum(II))-2-amino-1-hydroxyethane-1,1-diyl-bisphosphonate (A, Fig. 3)³⁹ and bis-(ethylenediamineplatinum(II))medronate (B, Fig. 3)³⁹ were prepared as previously reported.

The adsorption and release of the two platinum complexes were investigated with respect to the HAp and HAhc nanocrystals. The adsorption experiments were performed at 37 °C in an aqueous medium and monitored for a period of 140 hours. The adsorbed amount of platinum complex was determined by the difference between the initial Pt complex quantity and that contained in the supernatant solution as a function of time.

Fig. 4 shows the adsorption profiles of the platinum complexes A and B on HA nanocrystals, starting from a 1 mg/ml complex concentration in water.

The plots show that the maximum uptake of complex A was reached after about 24 hours for both types of HA nanocrystals. Typically, the percentages of complex A adsorbed onto the HA nanoparticles with respect to the initial complex in solution were about 96% and 89% for HAp and HAhc, respectively. This corresponds to about 150 mg of complex per g of HA. The slight difference in the adsorption percentage observed for the two different HA nanocrystals may be ascribed to the differences in their degrees of crystallinity and surface areas.

Fig. 4 also shows the adsorption profiles of the platinum complex B on HA nanocrystals, starting from a 1 mg/ml complex concentration in water. The adsorption kinetics show similar trends for the two kinds of nanocrystals. After 140 h interaction, the percentages of complex adsorbed onto the HA nanoparticles with respect to the initial complex in solution were 72%, and 65% for HAp and HAhc, respectively. This corresponds to about 100 mg of complex B per g of HA.

The comparison between the two platinum complexes clearly shows the greater affinity of complex A for both types of HA nanoparticles, in terms of both adsorption rate and total uptake.
The maximum uptake of complex A was reached after about 24 hours for both types of HA nanocrystals, whilst the uptake of complex B after 24 hours was significantly lower and reached a maximum value after about 140 hours. It is also worth noting that complex A reached a plateau, being almost totally absorbed after 20 hours, while complex B did not reach a steady state during the experimental period. Two water washings were performed in order to evaluate the physisorbed platinum complex with respect to the chemisorbed one. The following drug release experiments were then carried out only upon the complex fraction effectively linked to the apatite surface.

The chemisorbed complex A, mg per g of HA, was 16 and 12 for HApC and HAhc, respectively. On the other hand, the chemisorbed complex B, mg per g of HA, was 14 and 9 for HApC and HAhc, respectively. It appears that the loading is slightly greater for HApC which is characterized by lower crystallinity and higher surface area, both factors favoring adsorption. At the stage in which the physisorbed complex portion has been removed, the affinity differences between the two HAs become appreciable, highlighting the higher affinity of HApC with respect to HAhc. These data also indicate that the amount of chemisorbed drug is not very different for the two types of complexes while the physisorbed portion appears to be greater for complex A and to take place by a faster kinetic. This is probably a consequence of the overall positive charge of A which renders this species more prone to electrostatic interaction with the negatively charged HA surface, above all through the interaction of the amino group with the apatitic phosphate.

### Platinum complexes release from HA

The release experiments were performed at 37 °C in aqueous Hepes buffer (NaCl concentration in the range 0.18–0.20 mM) and monitored for a 7 day period. The release profiles of complexes A and B from HA nanocrystals are plotted in Fig. 5. The data are expressed in terms of M/Mtot, where Mtot is the chemisorbed complex. Thus, Mtot should correspond to the amount of complex effectively bound to the HA nanocrystals.
The complex A release profiles were similar for the two different kinds of HA nanocrystals and the total amounts of complex released from HAp and HAhc at day 7 are very similar, 55% and 60% of the chemisorbed quantities, respectively. In the same way, the complex B release profiles were similar for the two kinds of HA nanocrystals, the amounts of complex released from HAp and HAhc being 100% and 97% of the chemisorbed quantities, respectively. Overall, complex B exhibits an appreciably faster release rate and a greater released amount with respect to complex A from both types of HA nanoparticles. The smaller release of chemisorbed complex in the case of A deserves a comment. As will be shown in the next section, platinum is released as a Pt(en) chlorido or solvato species, while the bisphosphonate remains anchored to the HA matrix. The BP in the case of A contains a free aminic function and we believe that this, by coordination to platinum, can sequester one of the two leaving metallic residues.

Characterization of the released Pt species

In order to characterize the Pt species released from the two different kinds of HA nanocrystals, UV-Vis experiments (reported in Fig. 6) have been performed on complexes A and B and on the platinum precursor [PtCl\(_2\)(en)].

This latter compound shows an intense band at ca. 203 nm (spectrum c in Fig. 6), assignable to ligand to metal charge transfer transitions, and a very weak and broad band at ca. 300 nm assignable to metal d-d transitions.\(^{36,37}\) Complexes A and B UV-Vis spectra (which are reported in Fig. 6(a) and (b), respectively) are characterized by a very intense band at 225 nm, this latter band falling at lower energy with respect to that observed for [PtCl\(_2\)(en)].

The UV-Vis spectrum of the platinum species released from HA nanocrystals (d in Fig. 6) is similar to that of [PtCl\(_2\)(en)] indicating that the complex released is [PtCl\(_2\)(en)] (or related solvato species). For the sake of clarity, we have reported only the UV-Vis spectrum of the species released from HA nanocrystals loaded with complex A, it being very similar to that of the analogous experiment performed on complex B.

The displacement of the platinum metal core from the BP is caused by the entrance of the chloride ions into the Pt coordination sphere, a process that was previously observed after inclusion of complex A in calcium containing silica xerogels.\(^{29}\) This substitution is hence a common trend observed in these inorganic conjugates and is probably due to a synergistic effect between calcium ions present on the HA nanocrystal surface which anchor the bisphosphonate ligand (due to the high affinity of anionic bisphosphonates for Ca\(^{2+}\)) and the chloride (0.18–0.20 mM) present in the buffer used for the release experiments (chloride ions have better coordinating ability towards the platinum soft metal core than oxygen).

Calcium release from HA nanocrystals before and after Pt complex loading

The amounts of calcium ions released in Hepes buffer saline solution from pure HAp and HAhc nanocrystals as a function of time are plotted in Fig. 7. The calcium ion amounts released after 7 days were 3.3% and 2.7% of the total content for HAp and HAhc, respectively, coherently with the higher surface disorder present on the HApc with respect to the HAhc nanocrystals.

Fig. 8 reports the released mass percentages of calcium ions as a function of time for the two different kinds of HA nanocrystals loaded with complexes A and B. In the case of samples loaded with complex A the amounts of released calcium after 7 days were 4.4% and 3.8% of the total calcium content for HAhc and HAp, respectively. Thus it appears to be slightly greater for HAhc than for HAp. In contrast, the opposite behaviour is observed for complex B loaded HA nanocrystals, the amounts of released calcium ions for HAp and HAhc nanocrystals being 3.6%, and 2.4% of the total calcium, respectively.

In any case the amount of calcium released is rather small in agreement with the hydroxyapatite low solubility at neutral pH. Complex B loaded on HA nanocrystals seems to weakly reduce the HA nanocrystals dissolution. In contrast complex A increases the HA nanocrystals dissolution affecting more the
highly ordered surface of HAhc than the less ordered surface of HAp.

**Cytotoxicity assays**

In order to assess the platinum derivatives released from HA nanocrystals in a cell growth medium as well as their cytotoxic activity, both unmodified and Pt-adsorbed HA nanocrystals were tested for their cytotoxic properties towards human cervix carcinoma cells (HeLa), notoriously highly sensitive to platinum-based drugs. For comparing the cytotoxicities of complexes A and B, we referred to equal concentrations of platinum released in the medium. The Pt content released in the medium was quantified by ICP analysis. Cell viability after 72 h drug exposure (from MTT test) and Pt concentration (from ICP analysis) are summarized in Table 2.

Unloaded HA nanocrystals were non-toxic whereas the HA-adsorbed complexes A and B were more cytotoxic than the unmodified A and B compounds.

In order to better understand the origin of this difference and to identify the nature of the Pt complex released, the cytotoxicity of the bisphosphonate-free Pt complex ([PtCl₂(en)]) was also assessed. Four [PtCl₂(en)] concentrations corresponding to the exact Pt amounts found in the release media were examined. The results are strictly comparable and suggest that the active species is, in all cases, [PtCl₂(en)] (or related solvato species), thus fully confirming what was already deduced from the UV-Vis analysis.

**Conclusions**

The aim of this study was the set-up of new implantable biomimetic hydroxyapatite phases with anticancer performance. Therefore HA nanocrystals with different chemico-physical properties, in terms of morphology, degree of crystallinity and surface area, were synthesized. Considering that surface area and crystallinity are strictly correlated to the “in vivo” resorption, these matrices can respond to different clinical needs.

The adsorption of two different platinum complexes, A and B, onto the synthesized nanocrystals has been investigated. Both complexes contain a geminal bisphosphonate but complex A contains, in addition, a charged ammonium group. This structural difference dramatically affects the affinity of A and B towards HA nanocrystals. Complex A having a greater affinity for calcium phosphate nanocrystals. The presence of a net negative charge on the HA surface could account for the favoured adsorption of complex A, which is positively charged.

The complex portion which was only physisorbed (physically entrapped inside the network between apatite nanocrystals) was removed by careful washing, leaving bound to the HA nanocrystals the complex portion which is chemisorbed (strictly linked to the crystal surface). The total uptake of complex A was slightly higher than that of B, however the ratio between physisorbed and chemisorbed fractions is retained.

The slightly greater uptake of both platinum complexes onto HAp (both in terms of physisorption and chemisorption) is imputable to its higher surface area and disorder, directly connected to its lower crystallinity degree.

The release profiles of the platinum complexes from the HA nanoparticles follow an inverted trend (complex B > complex A) when compared with the adsorption process. Most probably the less effective desorption in the case of complex A could be due to the aminic group present on the BP which remains anchored to the HA matrix, coordinating and holding some of the Pt(en) residues.

UV-Vis analysis showed that the released species is the same for complexes A and B, and matches the behaviour of [PtCl₂(en)] in similar conditions, thus indicating that the platinum-bisphosphonate bonds have been broken.

Unmodified and HA-adsorbed Pt complexes were tested for their cytotoxicity towards human cervix carcinoma cells (HeLa). The HA-loaded Pt complexes were more cytotoxic than the unmodified compounds A and B and their cytotoxicity was comparable to that of [PtCl₂(en)] thus indicating a common active species. This result was also supported by the UV-Vis investigation.

The assumption that the platinum-bisphosphonate bonds are broken in the release process is sustained by previous works, where the strong link between the bisphosphonate moieties and the calcium ions has been highlighted and clarified.

The above results demonstrate that HA nanocrystals and anti-tumor drugs can be conjugated in such a way to yield a smart

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Table 2 Cytotoxicity profiles

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pt (µM)</th>
<th>Cell viability (%) ± S.D.</th>
<th>Compound</th>
<th>Pt (µM)</th>
<th>Cell viability (%) ± S.D.</th>
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<tr>
<td>HAp</td>
<td>0</td>
<td>99.98 ± 1.23</td>
<td>HAhc</td>
<td>0</td>
<td>99.24 ± 1.33</td>
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<tr>
<td>A</td>
<td>5.35</td>
<td>74.12 ± 2.21</td>
<td>A</td>
<td>6.03</td>
<td>73.23 ± 1.55</td>
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<tr>
<td>A loaded</td>
<td>5.35</td>
<td>57.21 ± 1.64</td>
<td>A loaded</td>
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<td>60.20 ± 3.22</td>
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<tr>
<td>HAp</td>
<td>[PtCl₂(en)]</td>
<td>5.35</td>
<td>60.14 ± 1.41</td>
<td>[PtCl₂(en)]</td>
<td>6.03</td>
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<tr>
<td>B</td>
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<td>57.01 ± 2.11</td>
<td>[PtCl₂(en)]</td>
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</table>

* S.D. = standard deviation.
bone filler delivery system, acting both as bone substitutes and as platinum drug releasing agents with the final goal of locally inhibiting the tumor re-growth and reducing the systemic toxicity. The one here described not only can ensure a prolonged release of active species but also improves the performance of the unmodified drug. Moreover, these results suggest the possibility of using the chemico-physical differences of HA nanocrystals, above all degree of crystallinity, crystal size and surface area, in order to strongly tailor the Pt complex release kinetics.

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Notes and references